Promotion of tumor growth in vivo by antimacrophage agents

Keller, R

Abstract: Various attempts were made to assess the role of the mononuclear phagocyte system in tumor resistance of rats in vivo. The growth of sc inoculated, weakly immunogenic, carcinogen-induced, syngeneic tumor cells was modestly reduced by ip injection and, more impressively, by local injection of peptone-induced, activated, nonimmune macrophages. A single iv injection of silica particles or carrageenan on the day of sc tumor cell inoculation greatly enhanced tumor growth. When these agents had been given a few days before or after tumor cell inoculation, the tumor-promoting efficiency was distinctly diminished or even cancelled. The enhancing effects of silica and carrageenan on tumor growth were nullified by the macrophage-stabilizing agent, poly-2-vinylpyridine N-oxide. To assess the in vivo consequences of silica administration, various cellular, biochemical, and functional macrophage parameters were determined at different intervals. Results indicated the complexity of events elicited after the mononuclear phagocyte system was damaged, which made the interpretation of such results difficult

DOI: https://doi.org/10.1093/jnci/57.6.1355

Promotion of Tumor Growth In Vivo by Antimacrophage Agents[1, 2]

R. Keller[3, 4]

ABSTRACT—Various attempts were made to assess the role of the mononuclear phagocyte system in tumor resistance of rats in vivo. The growth of sc inoculated, weakly immunogenic, carcinogen-induced, syngeneic tumor cells was modestly reduced by ip injection and, more impressively, by local injection of peptone-induced, activated, nonimmune macrophages. A single iv injection of silica particles or carrageenan on the day of sc tumor cell inoculation greatly enhanced tumor growth. When these agents had been given a few days before or after tumor cell inoculation, the tumor-promoting efficiency was distinctly diminished or even cancelled. The enhancing effects of silica and carrageenan on tumor growth were nullified by the macrophage-stabilizing agent, poly-2-vinylpyridine *N*-oxide. To assess the in vivo consequences of silica administration, various cellular, biochemical, and functional macrophage parameters were determined at different intervals. Results indicated the complexity of events elicited after the mononuclear phagocyte system was damaged, which made the interpretation of such results difficult.—J Natl Cancer Inst 57: 1355–1361, 1976.

Many in vitro studies indicate that, in addition to the basic role that is assigned to lymphocytes (in tumor resistance), macrophages activated by specific or nonspecific means also contribute to tumor resistance (*1*). Since presently no direct or effective way exists of depleting the host of macrophages, their function in tumor resistance in vivo remains obscure. However, considerable evidence, admittedly indirect, implies such a capacity for the macrophage (*2*). For example, in the *Nippostrongylus brasiliensis*-infected rat, tumor growth can either be suppressed or enhanced, depending on the timing of inoculation of tumor cells in relation to the parasite infection (*3*). In this (*4*) and other in vivo model systems (*5, 6*), suppression of tumor growth seems attributable to AM, and enhancement of tumor growth seems due to a macrophage-inactivating factor arising after a nematode infection (*7*). Moreover, the use of adjuvants of microbial derivation such as BCG, *Corynebacterium parvum*, or *Listeria monocytogenes* has been considerably expanded, and recent evidence attests to intralesional injection of the adjuvant being particularly effective in producing regression of established syngeneic tumors (*8–11*). As the responsiveness of tumors to these adjuvants does not parallel their immunogenicity, this effect is now considered more likely to reflect the extent to which tumors are infiltrated by macrophages. Still other observations imply an inverse relationship between the macrophage content of tumors and tumor regression (*12*) or metastases (*13*). Despite these distinctive approaches and the expanding magnitude of the effort, no convincing evidence exists that macrophages are *directly* involved in tumor resistance.

The function of macrophages in tumor resistance is explored further in the present study. Various measures known to diminish the functional capacities of macrophages in vitro were studied for their effect on in vivo tumor growth; these treatments led to markedly enhanced tumor growth.

MATERIALS AND METHODS

Animals.—Inbred female DA rats, 170–210 g, were used. They were maintained under conventional conditions.

In vivo experiments with tumors.—DMBA- and MCA-induced tumors were obtained and passaged in vivo and maintained in tissue cultures as described in (*14, 15*). The tumor cell cultures were incubated for 2–4 days at 37° C on mycoplasma agar and checked regularly for absence of mycoplasma contamination.

Preparation of tumor cell suspensions.—The sc inoculation of 2×10^6 tumor cells usually leads to a localized, excisable subcutaneous tumor within 10–16 days. After necrotic parts of the tumor had been removed, the tissue was minced with scissors and pressed through nylon tissue (Schweizer Beuteltuch Nybolt; 120–125, ASTM) into culture medium; cell viability was assessed with 2% trypan blue. The number of tumor cells inoculated refers to cells capable of excluding the dye (60–70% of total cells).

In vitro experiments with tumors.—For the assessment of cytostatic and cytocidal macrophage capacities, DMBA-induced DA rat tumor cells and RPMI 7932 human melanoma cells that were not contaminated with mycoplasma were used as target cells in vitro under the conditions described in (*15, 16*).

Preparation of macrophages.—Macrophage monolayers were prepared as described in (*14*). Peritoneal cells from untreated controls (RM) or those obtained 3 days after ip injection of 10 ml of 10% proteose peptone (AM) were utilized as the source of adherent cells. In most experiments, the peptone-induced peritoneal cells consisted of at least 95% cells with characteristics of macrophages [morphology, adherence, phagocytosis (*15*)]. For passive transfer studies, macrophages were removed from culture dishes with a rubber policeman, suspended in PBS (2×10^6 cells/ml), and injected immediately into syngeneic recipient rats.

Macrophage supernatants were obtained after 24-

ABBREVIATIONS USED: AM = activated macrophages; DMBA = 7,12-dimethylbenz[*a*]anthracene; MCA = 3-methylcholanthrene; RM = normal resting macrophages; PBS = phosphate-buffered saline; PVNO = poly-2-vinylpyridine *N*-oxide; SRBC = sheep red blood cells; TDR = thymidine; IU = international units.

[1] Received March 24, 1976; accepted May 10, 1976.

[2] Supported by grant 3.234.74 from the Swiss National Science Foundation.

[3] Immunobiology Research Group, University of Zürich, Schönleinstrasse 22, CH-8032 Zürich, Switzerland.

[4] I am grateful to Professor R. Wyler, Institute of Virology, University of Zürich, for the assessment of possible mycoplasma contamination of the cell lines, and to Dr. A. C. Allison, Clinical Research Centre, Harrow, England, and Dr. M. Reisner, Steinkohlenbergbauverein, Essen, Bundesrepublik Deutschland, for generous gifts of silica. I thank Dr. Maurice Landy, Schweizerisches Forschungsinstitut, Davos, Switzerland, for his assistance in reviewing the manuscript. The expert technical assistance of Miss R. Keist, Miss M. Marazzi, and Miss R. Ming is gratefully acknowledged.

hour culture of 10^7 macrophages in the presence of 200 μg heat-sterilized silica particles (Dörentrup Quartz #12; average diameter, 5 μ) and freed of silica by being centrifuged at 10,000×*g* for 30 minutes and passed through 0.3-μ Millipore filters.

Particulates for alteration of the mononuclear phagocyte system.—Silica particles were suspended in PBS and injected as indicated. Carrageenan (Sea Kem 21; Marine Colloids, Inc., Springfield, N.J.) was dissolved in saline by being heated to 100° C for 15 minutes in a water bath; rats were given 5 mg iv in 0.5 ml saline. Trypan blue (Chroma, Stuttgart, Federal Republic of Germany) was dialyzed for 48 hours against glass-distilled water, lyophilized, and resuspended at a concentration of 10 mg/ml in PBS. In the first experiments, rats were given either ip or iv injections of trypan blue, 40 mg at 24 hours and 10 mg at 3 hours before inoculation of tumor cells; then 10 mg trypan blue was injected sc weekly. In later experiments, rats were given a single sc injection of 20 mg trypan blue.

Assessment of macrophage depletion.—The effect of silica treatments on the total population of peritoneal cells and on the number of macrophages was assessed at various intervals; 0.5 ml of the peritoneal washout containing approximately 2×10^6 cells/ml was mixed with 1 drop of 1% neutral red, and the number of macrophages was determined by hemacytometer counts. At the same time, blood monocyte counts were assessed.

PVNO (Polysciences, Inc., Warrington, Pa.) was injected sc at a dose of 150 mg/kg dissolved in 0.5 ml saline.

Assessment of macrophage functional activities.—Phagocytosis was determined after macrophage monolayers had een incubated for 30 minutes at 37° C with 2×10^7 untreated versus opsonized SRBC in 2 ml medium; this process yielded an SRBC:macrophage ratio of approximately 20:1. After incubation, free SRBC were removed by intensive washing of the monolayers. The dishes were air dried, fixed in methanol, and stained with Giemsa; the cells were then counted.

Cytostasis was measured after target cells were cultured for 4 hours at a macrophage:target cell ratio of 10:1. The cells were exposed for 60 minutes at 37° C to 1 μCi [^{3}H]TDR/dish ([*methyl*-^{3}H]TDR; 5 Ci/mmole; The Radiochemical Centre, Amersham, Buckinghamshire, England) as described in (*14, 15*). Data are reported as residual proliferation (percent of control).

Cytolysis was determined on target cells that were prelabeled (2×10^6–10^7) with 0.05 μCi [^{14}C]TDR (500 μCi/mmole; The Radiochemical Centre) in 5 ml medium for 12 hours, then thoroughly washed, and added to macrophage monolayers. The cells were then incubated for 18 or 36 hours at 37° C, after which they were removed with a rubber policeman and centrifuged. After solubilization with hot perchloric acid, sediments and supernatants were enumerated separately in toluene:Triton X-100 (2:1) scintillation fluid containing 4 g 2,5-bis(5-t-butylbenzoxazol-a-yl)-thiophane/liter (*17*) in a Tracerlab (ICN Pharmaceuticals N.V., Tracerlab Instruments Division, 2800 Mechelen, Belgium) liquid scintillator, and the percentage of ^{14}C released was assessed.

Assessment of macrophage biochemical parameters.—The levels of various enzymes of peritoneal mononuclear phagocytes were measured at different intervals after ip injection of silica. Lactate dehydrogenase (EC 1.1.1.27; L-lactate: NAD oxidoreductase) was assayed by determination of the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm (#15948; Biochemica LDH Test Combination, Boehringer, Mannheim, Federal Republic of Germany). Acid phosphatase activity was measured with sodium *p*-nitrophenyl phosphate as substrate (#15988; Biochemica Test Combination, Boehringer, Mannheim). β-Glucuronidase (EC 3.2.131; β-D-glucuronide glucuronohydrolase) activity was assayed by the method of Talalay et al. (*18*). *N*-Acetyl-β-D-glucosaminidase was assayed by the method of Woollen et al. (*19*) with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma Chemical Co., St. Louis, Mo.) as substrate. All enzyme activities were expressed in IU and refer to 2×10^6 adherent peritoneal cells. The 6-hour incorporation of 0.15 μCi D-[^{14}C]glucosamine/ml (200 μCi/mmole; The Radiochemical Centre) into acid-insoluble material of 2×10^6 adherent peritoneal cells was also determined (*20*).

RESULTS

Influence of Passive Transfer of Macrophages on Tumor Growth

One way of assessing the possible role of macrophages in tumor growth in vivo is to supplement the host's own macrophage reserve by passive transfer of AM and ascertain the effect on an sc inoculum of weakly immunogenic carcinogen-induced tumor cells (tables 1, 2). Injection of peptone-induced AM, even at a site away from that of tumor cell inoculation, consistently, but modestly, diminished tumor growth (table 1). The reduction of tumor growth was more pronounced when tumor cells were first admixed with a majority of AM and then immediately inoculated (group 2 in table 2).

Effects of a Single Injection of Silica or Carrageenan on Host Resistance to Tumors

Silica treatment markedly enhanced tumor growth, but the timing was crucial for this effect. Rats were given a single injection of 10 mg silica particles in 0.5 ml saline into a tail vein on days −10, −5, −3, 0, or +3 of sc tumor cell inoculation. In several experiments, the administration of silica particles on day 0 consistently resulted in

TABLE 1.—*Effect of ip inoculation of AM on growth of MCA-induced subcutaneous tumors* [a]

Mean tumor weight (g) on day 17	
Controls (0.2 ml saline ip on day 0)	2×10^6 AM ip (in 0.2 ml saline on day 0)
14.2±1.4 [b]	9.8±1.6 [b]

[a] 5×10^6 MCA-induced tumor cells were inoculated sc on day 0.
[b] Each value represents the mean of 15 rats. Differences in tumor weight between controls and rats given AM were statistically significant ($P<0.001$).

marked acceleration of tumor growth (table 3); the number of tumor takes and, even more markedly, the mean tumor weights were considerably increased in these rats. Silica treatment on day −3, and particularly on days +3, −5, or −10, did not interfere with host resistance to the tumor (table 3). Treatment of rats with carrageenan caused an analogous transitory diminution of host resistance (table 4), but only when the agent was given on the same day as the tumor cell inoculation.

Effect of Repeated Injections of Silica

In a series of experiments, rats inoculated sc with varying numbers of DMBA-induced syngeneic tumor cells were given successive injections of silica. Such continued treatment with silica particles led to an even more marked decrease in host resistance to the tumor (text-fig. 1A–F). In animals given larger numbers of tumor cells (i.e., between 10^3 and 5×10^4), the time of tumor appearance was markedly shortened by silica treatment. Thus, in controls inoculated with 10^4 tumor cells, it took 24 days until the tumor was detectable in all animals; in the silica-treated rats, tumors had already appeared by day 11. As a consequence of the early appearance of tumors in silica-treated animals, their mean tumor weight, particularly during the early phase, was consistently greater than that of controls. In control rats, tumors regularly contained one or several necrotic foci, whereas the tumor tissue of silica-treated rats was free of necrosis. In many experiments with control groups inoculated with 10^2 to 5×10^2 tumor cells, as few as 30% to a maximum of 70% of the rats developed tumors within 2–3 months, whereas rats that had been given silica and 10^2 tumor cells *all* developed tumors within 15 days (text-fig. 1A–F).

TABLE 2.—*Effect of local inoculation of AM on growth of DMBA-induced subcutaneous tumors* [a]

Treatment	Number of tumor takes on day 21/total No. of animals	Mean tumor weight g
Group 1: DMBA (controls)	10/10	8.2±1.6
Group 2: AM+DMBA (10:1)	4/10	0.9±1.0
Group 3: AM-supernatant+DMBA	10/10	9.7±2.1

[a] 10^4 DMBA-induced DA rat tumor cells were inoculated sc on day 0. In group 2, tumor cells were mixed with AM (ratio, 1:10) in vitro and then inoculated sc. Animals of group 3 were inoculated with tumor cells and supernatant from 10^7 AM incubated for 24 hr with silica. These animals were given the same amount of silica-free supernatant on days 1, 2, and 3.

TABLE 3.—*Abrogation of tumor resistance in DA rats by silica particles*

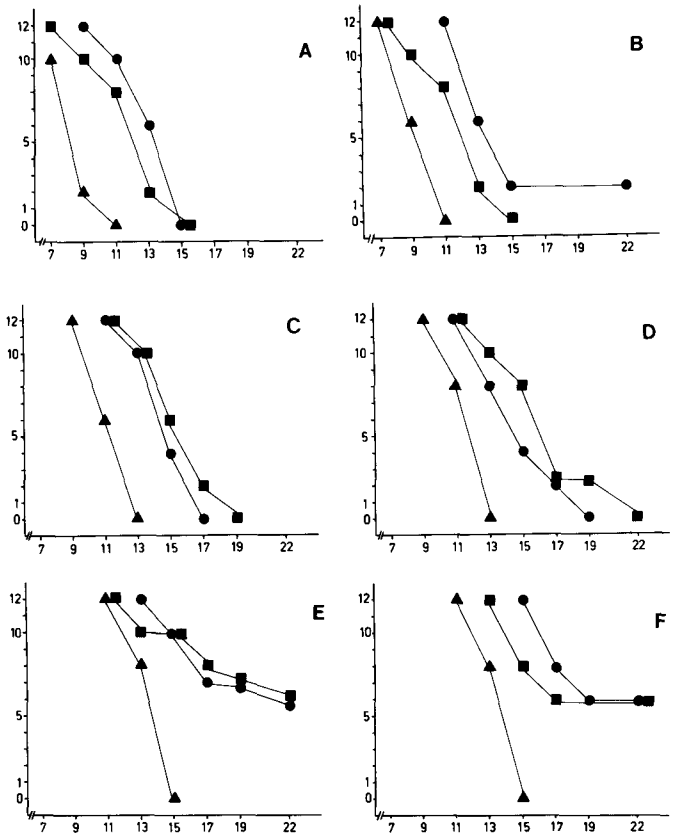
Time at which silica was given (10 mg/rat iv)	Tumor takes on day 15/total No. of animals	Mean tumor weight on day 15, g
Controls [a]	15/40	2.5±2.1
Day −10	7/10	1.9±0.9
Day −5	9/10	2.1±0.3
Day −3	30/30	6.5±5.3
Day 0	40/40	19.3±3.2
Day +3	13/30	2.5±0.7

[a] 10^4 DMBA-induced DA rat tumor cells were inoculated sc on day 0.

TABLE 4.—*Abrogation of tumor resistance on DA rats by carrageenan*

Time at which carrageenan was given (5 mg/rat iv)	Tumor takes on day 16/total No. of animals	Mean tumor weight on day 16, g
Controls [a]	21/30	5.2±3.1
Days −7, −5, −3	13/20	4.1±1.1
Day −3	17/30	4.6±2.1
Day 0	30/30	20.9±4.3
Day +3	28/30	5.3±3.0

[a] 10^4 DMBA-induced DA rat tumor cells were inoculated sc on day 0.



TEXT-FIGURE 1.—Enhancement of tumor growth by silica and its prevention by pretreatment with PVNO. *Ordinate*: Number of DA rats alive. *Abscissa*: Days after sc inoculation of DMBA-induced syngeneic tumor cells. ● = Controls (only tumor cells); ▲ = silica-treated rats; ■ = PVNO+silica. PVNO was injected sc on day −1 at a dose of 150 mg/kg; silica was injected as follows: day 0, 10 mg iv; days 1–3, 10 mg ip. 1A) 5×10^4 tumor cells sc on day 0; 1B) 10^4 tumor cells sc on day 0; 1C) 5×10^3 tumor cells sc on day 0; 1D) 10^3 tumor cells sc on day 0; 1E) 5×10^2 tumor cells sc on day 0; and 1F) 10^2 tumor cells sc on day 0.

Effect of Carrageenan on Tumor Resistance

A single iv injection of carrageenan the same day as tumor cell inoculation produced an analogous or even more pronounced enhancement of tumor growth. Tumors appeared earlier and for the early intervals, tumor weights were always higher than those in tumor-bearing controls (not shown). On the other hand, treatment of rats with silica or carrageenan did not visibly affect the growth characteristics of the tumor; i.e., there was no

evidence for an increased inclination to metastasis in the phase of early localized tumor growth. Combined treatment with silica and carrageenan had no cumulative tumor-enhancing effect (not shown).

Effect of Trypan Blue on Tumor Resistance

Hibbs (*21*) reported that trypan blue reduced tumor resistance in mice. However, the dosage of trypan blue he employed was toxic because 40–90% of the animals died within the first 5 days. In a typical experiment, 9 of 20 rats given combined injections of trypan blue iv and sc survived the period of acute toxicity, and all 9 developed large tumors (mean wt, 18.9 vs. 3.3 g in controls). In rats given a single sc injection of 20 mg trypan blue, which produced generalized bluing without signs of acute toxicity, tumor growth was not different from that in controls (not shown). Thus the source or type of trypan blue used may also be a factor in the outcome.

Prevention of Silica Effects by the Macrophage Stabilizer PVNO

PVNO, a potent macrophage-stabilizing agent, can diminish the fibrogenic and other in vivo effects of silica in animals (*22–26*). Therefore, the effect of PVNO pretreatment on the suppressive effects of silica and carrageenan on tumor resistance was examined. A single sc injection of PVNO on day −1 essentially nullified the tumor-enhancing effects of repeated administration of silica (text-fig. 1A–F). Abrogation of tumor resistance by carrageenan was also largely reversed by PVNO pretreatment of rats (not shown).

Effect of Silica on Peritoneal Macrophages and Blood Monocyte Counts in Normal Rats

To ascertain whether silica administration affected the total number of cells, and particularly the adherent mononuclear phagocytes present in peritoneal washouts, cell populations were examined at varying intervals after a single iv or ip injection of 10 mg silica/rat. Eight and 24 hours after local injection of silica particles, counts of total cells and peritoneal macrophages were increased rather than diminished (table 5); the counts then returned slowly to control levels. After ip administration of quartz, large phagolysosomes indicative of the ingestion of silica particles were regularly found in the cytoplasm of mononuclear phagocytes. The local inflammatory reaction to silica given ip was prevented by an sc injection of PVNO on the preceding day; this indicated that PVNO acted as a macrophage stabilizer in the present system. The injection of silica particles iv which produced no or only minor changes in the early phase was followed by a significant increase in the number of circulating monocytes that became evident after 3–4 days and that generally lasted for several days. On the other hand, silica given iv was not immediately followed by significant alterations in the peritoneal leukocyte population (table 5). Later on, the number of total peritoneal cells was generally slightly decreased.

Effects of Silica on Cytostatic and Cytocidal Capacities of Macrophages

Since the enumeration of blood monocytes and peritoneal macrophages can reflect only one aspect of silica administration, functional parameters such as phagocytosis and cytostatic and cytocidal capacity were also examined. These experiments showed that, after ip injection of 10 mg silica, phagocytosis of opsonized SRBC by adherent peritoneal cells was distinctly depressed for at least 4 days (not shown). Alterations in cytostatic and cytocidal peritoneal macrophage capacities after ip inoculation of silica are presented in tables 6 and 7. The data show that these distinctive capabilities are only weakly developed in untreated rats but markedly stimulated after local administration of peptone. However, in the early phase after local injection of silica particles, the cytocidal capacity of adherent peritoneal cells was even lower than that in untreated controls, i.e., for all practical purposes, absent. However, after 2–3 days, the cytostatic and cytocidal capacities of peritoneal cells developed and reached a degree comparable to that in peptone-treated rats. Even after 10–15 days of ip injection of silica, the cytostatic and destructive abilities of macrophages were markedly stimulated (not shown).

TABLE 5.—*Effect of systemic and local administration of silica particles on peritoneal cell populations (×10⁶)* [a]

Time after silica injection, hr	Silica, 10 mg ip [b]		Silica, 10 mg iv [b]	
	Total cells	Macrophages	Total cells	Macrophages
8	41.0±16 [c]	19.9±9.3 [c]	19.5±6.1	11.1±2.0
24	30.3±12 [c]	13.7±5.9 [c]	16.3±4.3	8.2±3.0
48	14.8±6.1	6.1±3.0	18.6±6.6	9.9±2.2
72	17.3±9.6	6.8±3.5	17.5±7.2	9.2±3.4
96	11.4±5.0	4.5±2.5	14.8±3.4	7.8±2.6

[a] In controls, counts were 14.3±5.7 for total cells and 6.7±2.7 for macrophages.
[b] Each value represents the mean of at least 20 determinations.
[c] Significantly different from controls ($P<0.001$).

TABLE 6.—*Effect of local administration of silica particles on macrophage cytostatic and cytocidal capacities* [a]

Adherent peritoneal cells sampled at various times after 10 mg ip silica injection, hr	Incorporation of [³H]TDR, % of control		Release of [¹⁴C]TDR [b], %	
	Target		Target	
	RPMI 7932	DMBA	RPMI 7932	DMBA
8	73±22	76±33	6±5	3±4 [c]
24	53±23 [c]	53±46	16±6	15±8
48	44±21 [c]	46±40 [c]	22±8 [c]	23±11 [c]
72	55±27	26±13 [c]	27±5 [c]	29±8 [c]
96	47±6 [c]	14±10 [c]	26±2 [c]	27±2 [c]
Adherent peritoneal cells from untreated controls (RM)	74±22	73±20	10±7	10±9
Peptone-induced adherent peritoneal cells (AM)	45±17 [c]	20±16 [c]	19±9 [c]	27±12 [c]

[a] The ratio of effectors (2×10^6) to targets (2×10^5) was 10:1. Each value represents the mean of at least 20 determinations, each performed in triplicate.
[b] Interaction was for 18 hr.
[c] These values were significantly different from controls ($P<0.001$).

TABLE 7.—*Effect of local administration of silica particles on biochemical macrophage parameters*[a]

Adherent peritoneal cells sampled at various times after 10 mg ip silica injection, hr	Lactate dehydrogenase	Acid phosphatase	β-Glucuronidase	N-Acetyl-β-glucosaminidase	Incorporation of [^{14}C]glucosamine, counts per minute	Incorporation of [^{3}H]TDR[b]	Percent release of [^{14}C]TDR within 18 hr[b]
8	47±12[c]	0.5±0.1[c]	0.4±0.2[c]	1.4±0.2[c]	202±128[c]	88±32	3±3
24	89±20	1.3±0.1	0.6±0.2[c]	2.8±0.3[c]	655±234	32±10[c]	10±3[c]
48	64±27	2.0±0.9	0.5±0.2[c]	2.9±0.8[c]	654±195	38±21[c]	13±8[c]
Adherent peritoneal cells from untreated controls (RM)	74±10	1.6±0.4	1.0±0.1	4.4±1	496±338	69±14	5±2
Peptone-induced AM	148±29[c]	6.6±2.0[c]	2.2±0.6	11.6±3	1,160±230	24±4	22±9

[a] 2×10^6 adherent peritoneal cells were used. Each value represents the mean of at least 15 determinations performed in triplicate. Enzyme activities were expressed in IU.

[b] RPMI 7932 (2×10^5 cells) was used as a target; ratio of AM to targets was 10:1. Incorporation of [^{3}H]TDR is always given in percentage of controls. Controls were target cells cultured in the absence of macrophages. Release of [^{14}C]TDR within 18 hr represents the experimental release [spontaneous release from prelabeled targets alone (= controls) has been deducted].

[c] Significantly different from controls.

Effects of Silica on Biochemical Capacities of Macrophages

The changes in biochemical parameters of peritoneal macrophages occurring at various intervals after ip administration of 10 mg silica are presented in table 7. In the early phase after silica administration, every examined parameter was clearly diminished; however, most had returned to normal within 24–48 hours. At these later intervals, cytostatic and cytolytic effects were again enhanced. The data indicate that the biochemical and functional activities of macrophages are differently affected by silica; some remain impaired for a prolonged period whereas others, after a brief initial decrease, soon reach values distinctly higher than those in resting controls (table 7).

Effect of Macrophage Supernatants on Tumor Growth

The present observations of a marked transitory decrease in tumor resistance in vivo after injection of various agents such as silica, carrageenan, and possibly trypan blue—all considered to interfere primarily with the mononuclear phagocyte system—could be explained in two different ways. First, macrophages that were damaged, functionally eliminated, or inhibited by these agents were no longer available for interaction with targets. Second, macrophage contents released by such agents might promote tumor growth. To assess the second possibility, silica-free supernatants from AM cultured for 24 hours in the presence of silica were repeatedly administered into the tumor site. In most experiments, such macrophage supernatants had weak growth-promoting effects (table 2). However, in two experiments in which the number of administered tumor cells was especially low, macrophage supernatants markedly increased the number of tumor takes (table 8).

TABLE 8.—*Enhancement of tumor growth by macrophage supernatants*[a]

Treatment	Tumor takes on day 30/total No. of animals
None (control)	2/16
Tumor cells mixed with AM (1:10)	1/16
Macrophage supernatants[b]	12/16

[a] 10^2 tumor cells were inoculated sc on day 0.

[b] 1 ml supernatant from 10^7 AM cultivated for 24 hr in the presence of 200 μg silica was mixed with tumor cells and injected sc on day 0. An additional 1 ml supernatant was injected each time into the tumor site 1, 2, and 3 days later.

DISCUSSION

Mononuclear phagocytes activated by specific or nonspecific means acquire the capacity to interact with many targets in vitro in various ways (*1, 27*); however, the in vivo implications of these interactions are still a matter of conjecture. The present work which shows that passively administered AM (ip, table 1, or sc with tumor cells, table 2) inhibit tumor growth also indicates an antitumor effect of AM. However, direct evidence has not yet been presented for macrophages as effectors of tumor resistance in vivo.

Since the host cannot be depleted of macrophages, it was necessary to resort to less decisive alternatives. Among these, an especially compelling alternative derives from the extraordinary selective susceptibility of macrophages in vitro to the cytotoxicity of silica particles (*28, 29*). Then, also, in vivo administration of silica is known to elicit major alterations in host resistance, e.g., prolonged survival of skin allografts (*26, 30*), abrogation of resistance to bone marrow grafts (*25*), suppression of established delayed hypersensitivity reactions (*31*), and depression of resistance to herpes simplex virus (*32, 33*) and yellow fever virus in mice (*34*). Moreover, silica pretreatment interferes with the establishment of active (*30, 35, 36*) and adoptive (*37*) antitumor immunity and actually induces lymphomatous tumors in Wistar rats (*38*). Despite the lack of knowledge of the character of the in vivo effects elicited by silica, these findings have been interpreted as a reflection of macrophage depletion.[5]

[5] Since the completion of the present work, Levy and Wheelock (*39*) reported on the immune and nonimmune functions altered by silica given iv. Their data show that, among various effects, depression of clearance of colloidal carbon and in vitro phagocytic activity for at least 3 days is the most uniform change; silica had no direct depressive effect on cells other than macrophages.

Carrageenan, a high-molecular-weight sulfated polygalactose obtained from marine plants, affects various biologic systems such as blood coagulation (*40, 41*) and the complement system (*42, 43*). In addition, carrageenan is toxic for macrophages in vitro (*29, 44*) and leads to inhibition of delayed hypersensitivity reactions (*31*) and suppression of primary antibody response and tolerance induction in vivo (*45, 46*). Trypan blue, an inhibitor of lysosomal enzyme activity (*47*), is readily taken up into secondary lysosomes but is not ingested by viable lymphocytes, neutrophils, basophils, or eosinophils (*48, 49*). Accordingly, this dye appears to be another useful experimental tool for the evaluation of the role of the macrophage in vivo (*10*). Apart from these agents, antimacrophage sera have repeatedly been shown to impair macrophage function both locally and systemically (*32, 50*). These observations created some optimism that agents such as silica, carrageenan, and trypan blue might provide the means for effecting a transient depletion—or at least a functional blocking—of the mononuclear phagocyte system in vivo.

The present study has shown, however, that such expectations were too high. Although silica does affect resistance to syngeneic tumors, these changes are not to be construed as simple or direct with regard to macrophages. Thus data such as those summarized in table 3 demonstrate that a single iv injection of silica particles on the day of tumor cell inoculation largely abrogates resistance of DA rats to a syngeneic DMBA-induced, weakly immunogenic tumor. This decrease in host resistance was especially conspicuous in rats inoculated with 10^2 or 5×10^2 tumor cells (text-fig. 1). However, silica treatment was marginally effective on days -3 or $+3$ and was noneffective on days -5 or -10 (table 3); the duration of the effect is thus sharply circumscribed. The efficacy of a single injection of carrageenan in abrogating tumor resistance was similarly pronounced and just as restricted to the day of tumor cell inoculation (table 4). Trypan blue, however, was without such tumor-enhancing effect. Although the underlying processes are not understood, it is noteworthy that large tumors excised from silica-treated animals seldom showed necrosis whereas tumors from controls always contained one or several necrotic foci.

As silica and carrageenan effectively promoted the growth of a syngeneic tumor of low immunogenicity, a depletion of host macrophages seemed the most direct mechanism by which these in vivo effects were achieved. However, cell counts (table 5) failed to reveal a decrease in the number of mononuclear phagocytes consistent with the observed changes in host resistance. On the other hand, all functional and biochemical parameters of the examined macrophages were distinctly diminished in the early phase after silica but often significantly enhanced at later intervals (table 6).

In principle, the present findings of a marked enhancement of tumor growth by silica and carrageenan could involve two essentially opposite mechanisms. The more obvious interpretation is that the brief period of clearly diminished macrophage function makes for successful tumor implantation and initiation of progressive tumor growth. However, an alternative explanation could be that tumor growth is simply enhanced by some trophic growth-promoting agent released from macrophages damaged by silica. Some support for this view is provided by the finding that macrophages and their culture supernatants do indeed exhibit growth-promoting activity in vitro (*14, 27, 51–53*). Preliminary findings indicate that supernatants from silica-treated macrophages are potent inducers of enhanced proliferation of tumor cells. These two opposing interpretations are each reasonably consistent with most of the present findings. For example, the reversal of the tumor-enhancing effect exerted by silica and carrageenan by the lysosome stabilizer PVNO, a compound that effectively reduces or prevents the toxic, fibrogenic, and immunosuppressive effects of silica (*22, 23, 25, 26, 29, 54*), would be consistent with either mechanism.

It seems reasonable that the well-documented toxicity in vitro of silica for macrophages is probably limited to the very early phases of its administration in vivo; quickly thereafter, the components released from damaged macrophages induce an inflammatory reaction that gives rise to a cascade of cellular and biochemical consequences. The functional decline in macrophages observed early after the administration of silica particles is followed by rapid regeneration, or even a considerable stimulation of important macrophage activities such as cytostatic and cytolytic capacities. The macrophage reserves of the host seem highly inexhaustible, and the paradoxical possibility should be considered that some constituents released by damaged macrophages may actually trigger or signal their migration from the bone marrow or perhaps even their local proliferation (*55, 56*). It is thus evident that extrapolation from the in vitro findings on silica cytotoxicity is misleading. Accordingly, this approach is unsuitable for appraising the contribution of macrophages to host resistance.

REFERENCES

(*1*) KELLER R: Cytostatic and cytocidal effects of activated macrophages. *In* Immunobiology of the Macrophage (Nelson DS, ed.), chapt 19. New York, Academic Press, 1976, pp 487–508

(*2*) LEVY MH, WHEELOCK EF: The role of macrophages in defense against neoplastic disease. Adv Cancer Res 20:131–163, 1974

(*3*) KELLER R, OGILVIE BM, SIMPSON E: Tumour growth in nematode-infected animals. Lancet 1:678–680, 1971

(*4*) KELLER R, JONES VJ: Role of activated macrophages and antibody in inhibition and enhancement of tumour growth in rats. Lancet 2:847–849, 1971

(*5*) KELLER R, HESS MW: Tumour growth and nonspecific immunity in rats: The mechanisms involved in inhibition of tumour growth. Br J Exp Pathol 53:570–577, 1972

(*6*) STIFFEL C, MOUTON D, BIOZZI G: Role of RES in the defense against invasion by neoplastic, bacterial and immunocompetent cells. *In* The Reticuloendothelial System and Immune Phenomena (di Luzio NR, Flemming K, eds.). New York, Plenum Press, 1971, pp 305–314

(*7*) KELLER R: Evidence for compromise of tumour immunity in rats by a non-specific blocking serum factor that inactivates macrophages. Br J Exp Pathol 54:298–305, 1973

(*8*) ZBAR B, TANAKA T: Immunotherapy of cancer: Regression of tumors after intralesional injection of living *Mycobacterium bovis*. Science 172:271–273, 1971

(*9*) LIKHITE VV, HALPERN BN: The delayed rejection of tumors formed from the administration of tumor cells mixed with

killed *Corynebacterium parvum*. Int J Cancer 12:699–704, 1973

(10) Bast RC, Zbar B, Mackaness GB, et al: Antitumor activity of bacterial infection. I. Effect of *Listeria monocytogenes* on growth of a murine fibrosarcoma. J Natl Cancer Inst 54:749–756, 1975

(11) Bast RC, Zbar B, Miller TE, et al: Antitumor activity of bacterial infection. II. Effect of *Listeria monocytogenes* on growth of a guinea pig hepatoma. J Natl Cancer Inst 54:757–761, 1975

(12) Russell SW, Doe WF, Cochrane CG: Number of macrophages and distribution of mitotic activity in regressing and progressing Moloney sarcomas. J Immunol 116:164–166, 1976

(13) Eccles SA, Alexander P: Macrophage content of tumours in relation to metastatic spread and host immune reaction. Nature 250:667–669, 1974

(14) Keller R: Cytostatic elimination of syngeneic rat tumor cells in vitro by nonspecific activated macrophages. J Exp Med 138:625–644, 1973

(15) ———: Modulation of cell proliferation by macrophages: A possible function apart from cytotoxic tumour rejection. Br J Cancer 30:401–415, 1974

(16) ———: Susceptibility of normal and transformed cell lines to cytostatic and cytocidal effects exerted by macrophages. J Natl Cancer Inst 56:369–374, 1976

(17) Keller R, Keist R, Ivatt RJ: Functional and biochemical parameters of activation related to macrophage cytostatic effects on tumour cells. Int J Cancer 14:675–683, 1974

(18) Talalay P, Fishman WH, Huggins C: Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrates for the assay of glucuronidase activity. J Biol Chem 166:757–772, 1946

(19) Woollen JW, Heyworth R, Walker PG: Studies on glucosaminidase. III. Testicular *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase. Biochem J 78:111–116, 1961

(20) Hammond ME, Dvorak HF: Antigen-induced stimulation of glucosamine incorporation in delayed hypersensitivity. J Exp Med 136:1518–1532, 1972

(21) Hibbs JB: Activated macrophages as cytotoxic effector cells. I. Inhibition of specific and nonspecific tumor resistance by trypan blue. Transplantation 19:77–81, 1975

(22) Holt PF: Poly(vinylpyridine oxides) in pneumoconiosis research. Br J Ind Med 28:72–77, 1971

(23) Schlipköter HW, Dolgner R, Brockhaus A: Ein Beitrag zur Therapie der experimentellen Silikose. Dtsch Med Wochenschr 88:1895–1899, 1963

(24) Barhad B, Rotaru G, Petrescu L, et al: Veränderungen "in vivo" der alveolären Koniophagen unter dem Einfluss des Siliciumdioxyds und des Polyvinylpyridin *N*-oxyds. Int Arch Gewerbepathol 24:148–153, 1967

(25) Lotzovà E, Cudkowicz G: Abrogation of resistance to bone marrow grafts by silica particles. Prevention of the silica effect by the macrophage stabilizer poly-2-vinylpyridine *N*-oxide. J Immunol 113:798–803, 1974

(26) Rios A, Simmons RL: Poly-2-vinylpyridine *N*-oxide reverses the immunosuppressive effects of silica and carrageenan. Transplantation 13:343–345, 1972

(27) Keller R: Cytostatic and cytocidal effects of activated nonimmune macrophages. *In* The Macrophage in Neoplasia. New York, Academic Press, 1976. In press

(28) Kessel RW, Monaco L, Marchisio MA: The specificity of the cytotoxic action of silica—a study in vitro. Br J Exp Pathol 44:351–364, 1963

(29) Allison AC, Harington JS, Birbeck M: An examination of the cytotoxic effects of silica on macrophages. J Exp Med 124:141–153, 1966

(30) Pearsall NN, Weiser RS: The macrophage in allograft immunity. I. Effects of silica as a specific macrophage toxin. J Reticuloendothel Soc 5:107–120, 1968

(31) Schwartz HJ, Lesokwitz S: The effect of carrageenan on delayed hypersensitivity reactions. J Immunol 103:87–91, 1969

(32) Zisman B, Hirsch MS, Allison AC: Selective effects of antimacrophage serum, silica and anti-lymphocyte serum on pathogenesis of herpes virus infection of young adult mice. J Immunol 104:1155–1159, 1970

(33) Hirsch MS, Zisman B, Allison AC: Macrophages and age-dependent resistance to herpes simplex virus in mice. J Immunol 104:1160–1165, 1970

(34) Zisman B, Wheelock EF, Allison AC: Role of macrophages and antibody in resistance of mice against yellow fever virus. J Immunol 107:236–243, 1971

(35) Wheelock EF, Toy ST, Weislow OS, et al: Restored immune and nonimmune functions in Friend virus leukemic mice treated with statolon. Prog Exp Tumor Res 19:369–389, 1974

(36) Erb P, Diethelm A, Löliger S, et al: Der Einfluss intraperitonäaler oder subkutaner Immunisierung mit Tumorzellmembranen auf die Entstehung eines Enhancement von durch Adenovirus Typ 12 induzierten Tumoren bei Hamstern. Schweiz Med Wochenschr 102:1186–1188, 1972

(37) Zarling JM, Tevethia SS: Transplantation immunity to simian virus 40-transformed cells in tumor-bearing mice. II. Evidence for macrophage participation at the effector level of tumor cell rejection. J Natl Cancer Inst 50:149–157, 1973

(38) Wagner MM, Wagner JC: Lymphomas in the Wistar rat after intrapleural inoculation of silica. J Natl Cancer Inst 49:81–91, 1972

(39) Levy MH, Wheelock EF: Effects of intravenous silica on immune and non-immune functions of the murine host. J Immunol 115:41–48, 1975

(40) Schwartz HJ, Kellermeyer RW: Carrageenan and delayed hypersensitivity. II. Activation of Hageman factor by carrageenan and its possible significance. Proc Soc Exp Biol Med 132:1021–1024, 1969

(41) Anderson W, Duncan JG: The anticoagulant activity of carrageenan. J Pharm Pharmacol 17:647–654, 1965

(42) Davies GE: Inhibition of complement by carrageenan: Mode of action, effect of allergic reactions and on complement of various species. Immunology 8:291–299, 1965

(43) Borsos T, Rapp HJ, Crisler C: The interaction between carrageenan and the first component of complement. J Immunol 94:662–666, 1965

(44) Catanzaro PJ, Schwartz HJ, Graham RC: Spectrum and possible mechanism of carrageenan cytotoxicity. Am J Pathol 64:387–404, 1971

(45) Lukić ML, Cowing C, Leskowitz S: Strain differences in case of tolerance induction to bovine γ-globulin: Dependence on macrophage function. J Immunol 114:503–506, 1975

(46) Bice DE, Gruwell DG, Salvaggio JE, et al: Suppression of primary immunization by carrageenan—a macrophage toxic agent. Immunol Communic 1:615–625, 1972

(47) Beck F, Lloyd JB, Griffiths A: Lysosomal enzyme inhibition by trypan blue: A theory of teratogenesis. Science 157:1180–1182, 1967

(48) Padawer J: The peritoneal cavity as a site for studying cell-cell and cell-virus interactions. J Reticuloendothel Soc 14:462–512, 1973

(49) Hibbs JB: Heterocytolysis by macrophages activated by bacillus Calmette-Guérin: Lysosome exocytosis into tumor cells. Science 184:468–471, 1974

(50) Stinnett JD, Kaplan AM, Morahan TS: Identification of a macrophage-specific cell surface antigen. J Immunol 116:273–278, 1976

(51) Keller R: Major changes in lymphocyte proliferation evoked by activated macrophages. Cell Immunol 17:542–551, 1975

(52) Calderon J, Unanue ER: Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. Nature 253:359–361, 1975

(53) Nathan CF, Terry WD: Differential stimulation of murine lymphoma growth in vitro by normal and BCG-activated macrophages. J Exp Med 142:887–902, 1975

(54) Marchisio MA, Comolli R: Studio metabolico e morfologico dell'attività litico-protettiva della poli-2-vinilpiridina-*N*-ossido su macrofagi peritoneali trattati con silice. Med Lav 55:401–410, 1964

(55) Wynne KM, Spector WG, Willoughby DA: Macrophage proliferation in vitro induced by exudates. Nature 253:636–637, 1975

(56) Adolphe M, Fontagne J, Pelletier M, et al: Induction of DNA synthesis in rat macrophages in vitro by inflammatory exudate. Nature 253:637, 1975